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14. ABSTRACT

The objectives of this proposal are to determine whether protein kinase C epsilon (PKCε) is linked to the initiation and progression of Prostate cancer (PCa) and should be explored as a molecular target for the prevention of human PCa. PKCε, a calcium-insensitive PKC, is among the PKC isoforms expressed in both mouse and human prostate tissue. We plan to test the hypothesis that PKCε is linked to the onset, progression and metastasis PCa. Two specific aims are proposed to test this hypothesis. Specific Aim #1: To obtain the first molecular genetic evidence that PKCε is linked to the development of PCa. To accomplish this specific aim, we will employ TRAMP mice, the well established mouse model of PCa. We will deplete PKCε in TRAMP mice by crossbreeding TRAMP mice with PKCε knockout (-/-) mice. We will evaluate TRAMP-PKCε KO mice for the development and progression of PCa *in vivo*. We will determine whether the genetic loss of one (-/+) or both (-/-) PKCε alleles will attenuate the progression of PCa. Specific Aim #2: To explore the mechanisms by which PKCε may promote the progression of AI PCa. This report will review the accomplishments made over the first year of grant award with respect to these specific objectives and according to the time line proposed in the original statement of work of the project.

15. SUBJECT TERMS

Prostate Cancer, androgen-dependent, androgen-independent, Protein kinase C epsilon, transgenic adenocarcinoma of mouse prostate, interleukin

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ANNUAL REPORT FOR AWARD NUMBER " W81XWH-07-1-0049"
ENTITLED MOLECULAR TARGETS FOR PREVENTION OF PROSTAT CANCER.
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INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer-related deaths in men. The risk of PCa increases rapidly after age 50 in men, with two-thirds of all PCa cases found in men after age 50. PCa first manifests as an androgen-dependent (AD) disease and can be treated with androgen-deprivation therapy. Despite the initial success of androgen ablation therapy, PCa progresses from AD to androgen-independent (AI). The hormone refractory, invasive PCa is the end stage and accounts for the majority of PCa patient deaths. Defining the molecular mechanisms linked to the transition of AD PCa to a hormone refractory PCa is essential in planning strategies in the prevention and treatment of PCa. The objectives of this proposal are to determine whether protein kinase C epsilon (PKCε) is linked to the initiation and progression of Prostate cancer (PCa) and should be explored as a molecular target for the prevention of human PCa. PKC represents a family of Phospholipid-dependent, serine/threonine protein kinases. PKCε is a calcium-insensitive PKC. Previous studies have shown, using cultured prostate cancer-derived cell lines and human PCa specimens that PKCε may play a role in the progression to AI PCa. However, the role PKCε plays in the course of PCa progression on the whole tissue level *in vivo* is unknown and that forms the focus of this proposal. We plan to test the hypothesis that PKCε is linked to the onset, progression and metastasis PCa. Two specific aims are proposed to test this hypothesis: Specific Aim #1: To obtain the first molecular genetic evidence that PKCε is linked to the development of PCa. To accomplish this specific aim, we will employ TRAMP mice. Specific Aim #2: To explore the mechanisms by which PKCε may promote the progression of AI PCa. PKCε may be a new marker for the prognosis of PCa, as well as a molecular target for the prevention and therapy of PCa. Knowledge obtained from the proposed study will help to plan strategies to manage the development of PCa.
This report will review the accomplishments made over the second year of grant award with respect to these specific objectives and according to the time line proposed in the original statement of work of the project.

BODY (Key Research Accomplishments by original statement of work)

***Task 1*: Specific Aim #1: To obtain the first molecular genetic evidence that PKCε is linked to the development of PCa**. **Anticipated time to accomplish: 18-28 months**

The principle experimental approach to link PKCε to the development of PCa is to deplete PKCε in TRAMP mice. This will be accomplished by crossbreeding

TRAMP mice with PKCε knockout (-/-) mice. We will evaluate TRAMP-PKCε KO mice for the development and progression of PCa *in vivo.* We will determine whether genetic loss of one (-/+) or both (-/-) PKC alleles will attenuate the progression of PCa. Our PKCε knockout (-/-) mice are on FVB background while TRAMP mice were on C57BL/6 background. This year, we were successful to obtain TRAMP mice on FVB background from Dr. Barbara Foster's laboratory, Rosewell Park cancer Institute, Buffalo, New York. Congenic FVB TRAMP mice were originally generated by Dr. Allan Balmain's group as follows: B6 TRAMP females were mated with FVB males to generate B6FVBF1 TRAMP animals and the F1 TRAMP females were backcrossed to FVB males; this scheme of backcrossing TRAMP females to FVB males was continued. The FVB TRAMP mice to be used in this study will be generation N9-N12. The

study of the influence of genetic background in prostate pathology between FVB and B6 TRAMP mice reveals that FVB mice have a significantly higher incidence of malignant neuroendocrine (NE) carcinomas and a significantly shorter survival time (20 weeks) compared to B6 mice (52 weeks). All FVB mice developed malignant NE carcinomas by 20 weeks compared to a lifetime incidence of 20% in B6 mice. Since FVB TRAMP mice have median survival of 20 weeks, a time course study will be performed with 4, 8, 12, 16, and 20 weeks old mice.

We are currently breeding FVB PKCε knockout (-/-) with FVB TRAMP mice to generate sufficient mice on each genotype (TRAMP+, PKCε +/+); (TRAMP+, PKCε -/+); and (TRAMP+, PKCε -/-) for the proposed experiments as illustrated below. We anticipate to complete these experiments in the last year of our grant period.

Experiment 1: **Effects of PKCε deletion on the development of PCa in TRAMP mice.** All mice will be randomly assigned to the indicated cohort and sacrificed at 4, 8, 12, 16, and 20 weeks of age. There will be 20 mice per experimental group. Since it is difficult to generate sufficient numbers of male mice for all the time points, we have divided this experiment in three separate parts.

Experiment 1A: The link of PKCε to the progression to AI PCa. In this experiment, there will be 40 male mice in each genotype (TRAMP+, PKCε +/+); (TRAMP+, PKCε -/+); and (TRAMP+, PKCε -/-). At 8 weeks of age, 20 mice of each genotype will be castrated. For castration, mice will be anesthetized with sodium pentobarbital (65 mg/kg, administered i.p.) and an incision will be made across the lower abdomen to allow access to the testes. The ductus deferens will then be cauterized and the testes removed. The incision will be closed by staples, which will then be removed two weeks post operation. At 20 weeks of age, all mice will be sacrificed.

Cohorts	**Sacrificed at: 20 weeks**	
	Intact	**Castrated**
TRAMP/PKCε (+/+)	20	20
TRAMP/PKCε (+/-)	20	20
TRAMP/PKCε (-/-)	20	20

Experiment 1B: The link of PKCε to the initiation of PCa. In this experiment, 20 male mice of each genotype will be sacrificed at 4 and 8 weeks of age to determine whether PKCε deletion prevents the development of early lesion (PIN) in TRAMP mice.

Cohorts	**All intact, sacrificed at:**	
	4 weeks	**8weeks**
TRAMP/PKCε (+/+)	20	20
TRAMP/PKCε (+/-)	20	20
TRAMP/PKCε (-/-)	20	20

Experiment 1C: The link of PKCε to the development of PCa at the post-initiation phase of prostate carcinogenesis. In this experiment, mice of each genotype will be sacrificed at 12, 16, and 20 weeks of age.

Cohorts	**All intact, sacrificed at:**		
	12 weeks	**16weeks**	**20 weeks**
Experiment with	20	20	20
TRAMP/PKCε (+/+)	20	20	20
TRAMP/PKCε (+/-)	20	20	20
TRAMP/PKCε (-/-)			

Task 2: **Specific Aim #2: To explore the mechanisms by which PKCε may promote the progression of AI PCa. Anticipated time to accomplish: 24-36 months**

The proposed experimental approach was to determine the effects of the inhibition of PKCε expression in TRAMP mice, using the mice in Experiment 1 of Specific Aim #1, on the induction of cytokine IL-6 release and IL-6's associated signal transduction pathway (JAK/STAT-3) as well as the cell survival genes (e.g., p21, p27, Survivin, Bcl-xL, and Bcl-2). The serum and prostate tissue samples will be collected from the

experiments outlined under Specific Aims #1. These samples will be used to analyze the level of expression of cytokine IL-6 using the mouse IL-6 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). The activation of the JAK/STAT-3 pathway and the expression of cell survival genes will be determined by Western blot analysis.
While we are waiting for the experiments to be accomplished under specific aim#1, we screened for pharmacological inhibitors of PKCε. The use of pharmacological inhibitors of PKCε will further provide clues of the role of PKCε in PCa development. We found that plumbagin, a medicinal plant-derived naphthoquinone, inhibits PKCε expression and growth and invasion of hormone refractory prostate cancer (**see Cancer Research paper in the appendix, this work was supported <u>in part</u> from DOD grant**). Our results with plumbagin are summarized below:

We found that plumbagin (PL), a quinoid constituent isolated from the root of the medicinal plant *Plumbago zeylanica L,* may be a potential novel agent in the control of hormone refractory PCa. Specific observations are the findings that PL inhibited PCa cell invasion and selectively induced apoptosis in PCa cells but not in immortalized non-tumorigenic prostate epithelial RWPE-1 cells. Also, intra-peritoneal administration of PL (2mg/kg body weight), beginning 3 days after ectopic implantation of hormone refractory DU145 PCa cells, delayed tumor growth by 3 weeks and reduced both tumor weight and volume by 90%. Discontinuation of PL treatment in PL- treated mice, for as long as 4 weeks did not result in progression of tumor growth. PL, at concentrations as low as 5 μM, inhibited both in cultured PCa cells and DU145 xenografts the expression of: 1) PKCε, PI3K, pAKT, pJAK-2 and pStat3; 2) the DNA-binding activity of transcription factors AP-1, NFkB, and Stat3 and 3) Bcl-xL, cdc25A and COX-2 expression. The results indicate for the first time, using both *in vitro* and *in vivo* preclinical models, that PL inhibits the growth and invasion of PCa. PL inhibits multiple molecular targets including PKCε, a predictive biomarker of PCa aggressiveness. PL may be a novel agent for therapy of hormone refractory PCa

KEY RESEARCH ACCOMPLISHMENT

1. To accomplish the proposed experiments under Specific Aim#1, we have now both TRAMP and PKCε KO mice on FVB background. Breeding of PKCε knockout (-/-) with TRAMP mice is in progress to generate sufficient mice on each genotype (TRAMP+, PKCε +/+); (TRAMP+, PKCε -/+); and (TRAMP+, PKCε -/-) for the proposed experiments.
2. Plumbagin, a natural inhibitor of PKCε, inhibits the growth and invasion of PCa.

PLANS: We will continue our experiments proposed under specific aims in the grant proposal. We anticipate no change in our original plans.

REPORTABLE OUTCOMES

A publication in CANCER RESEARCH (**Cancer Res. 68: 9024-9032, 2008).**

Patents and licenses – NONE

Degrees obtained – NONE

Development of cell lines, tissue or serum repositories – NONE

Informatics – NONE

Funding applied for based on work supported by this award : NONE

Employment or research opportunities applied for – NONE

CONCLUSIONS

Prostate cancer is the most common type of cancer in American men and ranks second to lung cancer in cancer-related deaths. While 1 in 6 men will get prostate cancer during his lifetime, 1 in 34 will die of this disease. Prostate epithelial cells are dependent on the male hormone androgen for survival and enter programmed cell death following hormone oblation resulting in involution of the prostate gland. Early PCa is typically diagnosed as androgen-dependent and is treated with anti-androgen drugs or using a procedure termed castration, which involves removal of the androgen producing testes. Despite androgen therapy, some of the cancer cells still survive and grow to form PCa. The PCa that grows after hormone therapy is called androgen independent (AI) PCa. This invasive PCa is the end stage and accounts for the majority of PCa patient deaths. The management of locally advanced prostate cancer is difficult and complex because the cancer often becomes hormone-insensitive and unresponsive to current chemotherapeutic agents. Knowledge about the regulatory molecules involved in the transformation to AI prostate cancer is essential for the rational design of agents to prevent and treat prostate cancer. Recently we found a protein termed protein kinase C epsilon (PKCε), which may play a role in the formation of advanced prostate cancer. The level of this protein is increased in prostate cancer tissue as compared to the normal prostate. The proposed study is aimed at validating the role of this protein in the progression of prostate cancer. Knowledge obtained from the proposed study will help to plan strategies to manage the development of PCa. This PKCε protein may be a new marker for the prognosis of PCa, as well as a molecular target for the prevention and therapy of PCa.

REFERENCES: None

APPENDICES:

1. **Cancer Research paper**

Research Article

Plumbagin, a Medicinal Plant–Derived Naphthoquinone, Is a Novel Inhibitor of the Growth and Invasion of Hormone-Refractory Prostate Cancer

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Abstract

Prostate cancer (PCa) is the second leading cause of cancer-related deaths in men. Hormone-refractory invasive PCa is the end stage and accounts for the majority of PCa patient deaths. We present here that plumbagin (PL), a quinoid constituent isolated from the root of the medicinal plant *Plumbago zeylanica L.*, may be a potential novel agent in the control of hormone-refractory PCa. Specific observations are the findings that PL inhibited PCa cell invasion and selectively induced apoptosis in PCa cells but not in immortalized nontumorigenic prostate epithelial RWPE-1 cells. In addition, i.p. administration of PL (2 mg/kg body weight), beginning 3 days after ectopic implantation of hormone-refractory DU145 PCa cells, delayed tumor growth by 3 weeks and reduced both tumor weight and volume by 90%. Discontinuation of PL treatment in PL-treated mice for as long as 4 weeks did not result in progression of tumor growth. PL, at concentrations as low as 5 μmol/L, inhibited in both cultured PCa cells and DU145 xenografts (*a*) the expression of protein kinase Cε (PKCε), phosphatidylinositol 3-kinase, phosphorylated AKT, phosphorylated Janus-activated kinase-2, and phosphorylated signal transducer and activator of transcription 3 (Stat3); (*b*) the DNA-binding activity of transcription factors activator protein-1, nuclear factor-κB, and Stat3; and (*c*) Bcl-xL, cdc25A, and cyclooxygenase-2 expression. The results indicate for the first time, using both *in vitro* and *in vivo* preclinical models, that PL inhibits the growth and invasion of PCa. PL inhibits multiple molecular targets including PKCε, a predictive biomarker of PCa aggressiveness. PL may be a novel agent for therapy of hormone-refractory PCa. [Cancer Res 2008;68(21):9024–32]

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer among men and is the second leading cause of cancer-related deaths (1). The risk of PCa increases rapidly after age 50, with two thirds of all PCa cases found in men after age 50. PCa first manifests as an androgen-dependent (AD) disease and can be treated with androgen deprivation therapy. Despite the initial success of androgen ablation therapy, PCa progresses from AD to androgen independent (AI). The hormone-refractory invasive PCa is the end stage and accounts for the majority of PCa patient deaths (2–6). At present, there is no effective treatment for AI metastatic PCa. There is an urgent need for novel agents that can be effective and selective in the prevention and treatment of hormone-refractory PCa. Plumbagin (PL), a medicinal plant–derived naphthoquinone (7), seems to possess such properties.

PL (5-hydroxy-2-methyl-1,4-napthoquinone; Fig. 1*A*) was isolated from the roots of the medicinal plant *Plumbago zeylanica L.* (also known as Chitrak; ref. 7). The roots of *Plumbago zeylanica* have been used in Indian medicine for more than 2,500 years for treatments of various ailments. PL is also present in black walnut and other various medicinal plants (7). PL has been shown to exert anticancer and antiproliferative activities in animal models and in cell culture (7). PL, fed in the diet (200 ppm), inhibits azoxymethane-induced intestinal tumors in rats (8). PL inhibits ectopic growth of breast cancer MDA-MB-231 cells (9), non–small cell lung cancer A549 cells (10), and melanoma A375-S2 cells in athymic nude mice (11). PL has also been shown to induce apoptosis in human PCa cell lines (12). However, no study exists about the effects of PL in the prevention and/or treatment of PCa progression.

We present in this communication for the first time that PL is a novel inhibitor of the growth and invasion of hormone-refractory PCa cells. I.p. administration of PL reduced both the weight and volume of ectopically xenografted DU145 cells by 90%. PL inhibited PCa cell invasion and selectively induced apoptosis in PCa cells. PL inhibited constitutive expression of multiple molecular targets, including protein kinase Cε (PKCε), phosphatidylinositol 3-kinase (PI3K), AKT, and activation of transcription factors activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and signal transducer and activator of transcription 3 (Stat3) in PCa cells. PL may be a novel agent for therapy of hormone-refractory PCa.

Materials and Methods

Chemicals, antibodies, and assay kits. PL (practical grade, purity >95%) was purchased from Sigma-Aldrich. The sources of antibodies used in this study were as follows: PKCε, other PKC isoforms, Stat3, phosphorylated Stat3Tyr705, PI3K (p85), PI3K (p110), p21, p27, vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9), Bcl-xL, cyclooxygenase-2 (COX-2), cdc25A, and β-actin (Santa Cruz Biotechnology); phosphorylated Janus-activated kinase (pJAK)-1 ($Tyr^{1022/1023}$), pJAK-2 ($Tyr^{1007/1008}$), phosphorylated AKT (pAKT; Ser^{473}), pAKT (Thr^{308}), and AKT (Cell Signaling Technology); pStat3Ser727 (BD Biosciences); and proliferating cell nuclear antigen (PCNA; Dako North America, Inc.). The oligonucleotides for AP-1 (5′-CGCTTGATGACTCAGCCGGAA-3′), NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′), and Stat3 (5′-GATCCTTCTGGGAATTCCTAGATC-3′) were obtained from Santa Cruz Biotechnology. Collagen-Based Cell Invasion Assay kit was from Millipore.

Cell lines. Cell lines (RWPE-1, CWR22rv1, LNCaP, PC-3, and DU145) were obtained from the American Type Culture Collection.

Apoptosis. Percent of cells undergoing apoptosis was determined by flow cytometric analysis of propidium iodide–stained cells (13).

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Cell invasion assay. Cell invasion was assayed using a Collagen-Based Cell Invasion Assay kit as per the manufacturer's instructions (14). Briefly, PCa cell lines at 80% confluency were serum starved for 18 to 24 h before the assay. The cells were harvested and the pellet was gently resuspended in serum-free medium. In the upper chamber, 0.5×10^6 cells per well were plated in triplicates and incubated for 2 h at 37°C in a humidified incubator with 5% CO_2 before PL treatment. Both the insert and the holding well were subjected to the same medium composition with the exception of serum. The insert contained no serum, whereas the lower well contained 10% fetal bovine serum that served as a chemoattractant. The untreated groups were used as a control. Forty-eight hours after PL treatment, the cell invasion assay was performed as per the manufacturer's instructions. The cells in the insert were removed by wiping gently with a cotton swab. Migrated cells sticking to the bottom side of the insert were stained with Cell Stain. Invading cells on the bottom side of the membrane were photographed using light inverted microscopy (Nikon Eclipse TS 100) at ×40 magnification. In addition, the number of cells migrating to the bottom side was estimated by colorimetric measurements at 560 nm according to assay instructions. Mean ± SE was calculated from three independent experiments.

Ectopic DU145 tumor xenografts. Male athymic nude mice were purchased from The Jackson Laboratory and raised in a pathogen-free environment. Mice were used for experimentation 2 wk after acclimatization. DU145 cells (2.5×10^6 in Matrigel) were implanted on both flanks of nude mice. The animals ($n = 10$) were treated with PL (2 mg/kg body weight in 0.1 mL PBS, 5 d a week) by i.p. injection 3 d after cell implantation. The untreated animals ($n = 10$) were used as a control. Mice were weighed and examined twice weekly for the presence of palpable tumors. Tumor size was measured by calipers and recorded. Tumor volume (V) was determined by the following equation: $V = (L \times W \times H \times 0.5236)$, where L is the length, W is the width, and H is the height of the xenograft tumor. At the end of study, mice were euthanized and digital photographs were taken of their tumors. The mean calculated tumor volume was plotted as a function of time. After 11 wk, PL treatment was stopped and the growth of the tumor was measured through 16 wk after cell implantation.

Statistical analysis. Statistical differences between the tumor volume means of control and PL-treated mice were analyzed by Student's t test.

Western blot analysis. Human PCa cells and xenograft samples were lysed in immunoprecipitation lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 200 mmol/L Na_3VO_4, 200 mmol/L NaF, 1 mmol/L EGTA]. The homogenate was centrifuged at 14,000 × g for 30 min at 4°C. Whole-cell lysate (25 μg) was fractionated on 10% to 15% SDS-polyacrylamide gels. The proteins were transferred to Hybond-P polyvinylidene difluoride transfer membrane (Amersham). The membranes were then incubated with the indicated primary antibodies followed by a horseradish peroxidase (HRP) secondary antibody and developed with Amersham enhanced chemiluminescence reagent and autoradiography using BioMax film (Kodak Co.). The Western blot signals were quantitated by densitometric analysis using TotalLab Nonlinear Dynamic Image analysis software (Nonlinear USA, Inc.).

Histology. Xenograft samples were fixed for 24 h in 10% neutral buffered formalin, transferred to PBS (pH 7.4), and then embedded in paraffin. Sections (4 μm thickness) of each specimen were cut for histologic and immunohistochemical examination.

Immunohistochemical analysis. Immunohistochemistry was carried out with rabbit anti-PKCε (1:200 dilution), rabbit anti-Stat3 (1:150 dilution), or mouse anti-PCNA (1:150) antibody in a Lab Vision Autostainer 3600 and PT module (Lab Vision) with a standard protocol for immunohistochemistry (14). Briefly, the samples of xenograft tumor were deparaffinized and antigen retrieval was done by heating in citrate buffer (pH 6.0; Lab Vision) at 98°C for 20 min and then incubated in peroxidase for 5 min to block endogenous peroxidase. Nonspecific proteins were blocked with Biocare Medical Terminator (Biocare Medical) for 10 min, and then samples were incubated with appropriate primary antibody at room temperature for 60 min followed by HRP-labeled IgG secondary antibody (Biocare Medical) for 40 min. Color was developed by incubating samples with diaminobenzidine (DAB)+ (Dako North America) for 1 min. CAT Hematoxylin (Biocare Medical) was used for 1 min as a counterstain. The specific staining of PKCε, Stat3, or PCNA in the sections was examined using

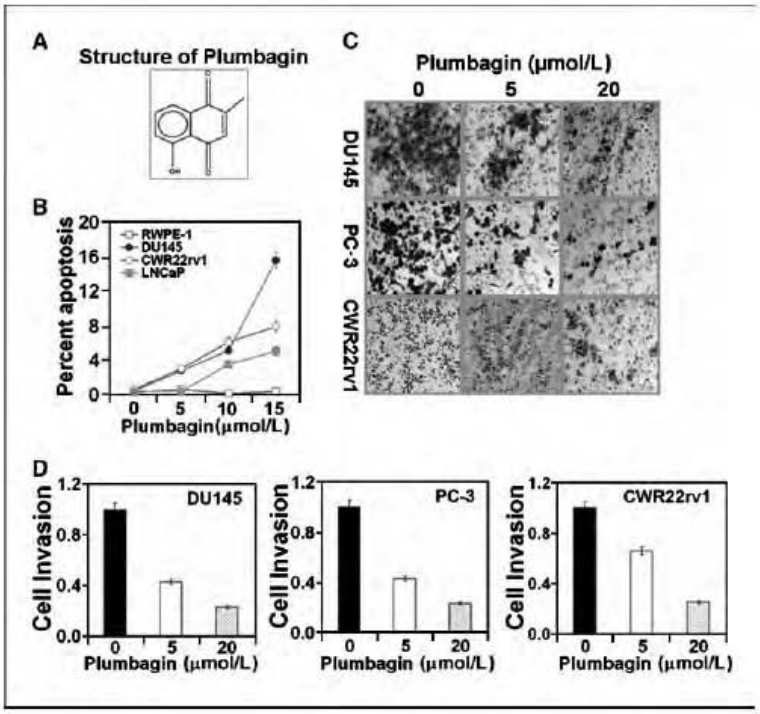

Figure 1. PL induces apoptosis and inhibits cell invasion in PCa cells. PCa cell lines (DU145, CWR22rv1, LNCaP, and RWPE-1) at 70% to 80% confluency were serum starved for 24 h and then treated with PL at various (0, 5, 10, and 15 μmol/L) concentrations in DMSO (final concentration, 0.1%). At 24 h after treatment, cells were collected for apoptosis analysis. CWR22rv1, DU145, and PC-3 cells were treated with 5 or 20 μmol/L of PL in DMSO (final concentration, 0.1%) for 48 h and assayed cell invasion as described before (14). *A*, structure of PL. *B*, induction of apoptosis. *Points*, mean of three separate dishes; *bars*, SE. *C*, PCa cell invasion. Cells were stained with crystal violet and photographed at ×40 magnification. *D*, number of invading cells was estimated by colorimetric measurements at 560 nm according to assay instructions (Chemicon International). *Columns*, mean of three separate wells; *bars*, SE. Similar results were observed in a repeat experiment.

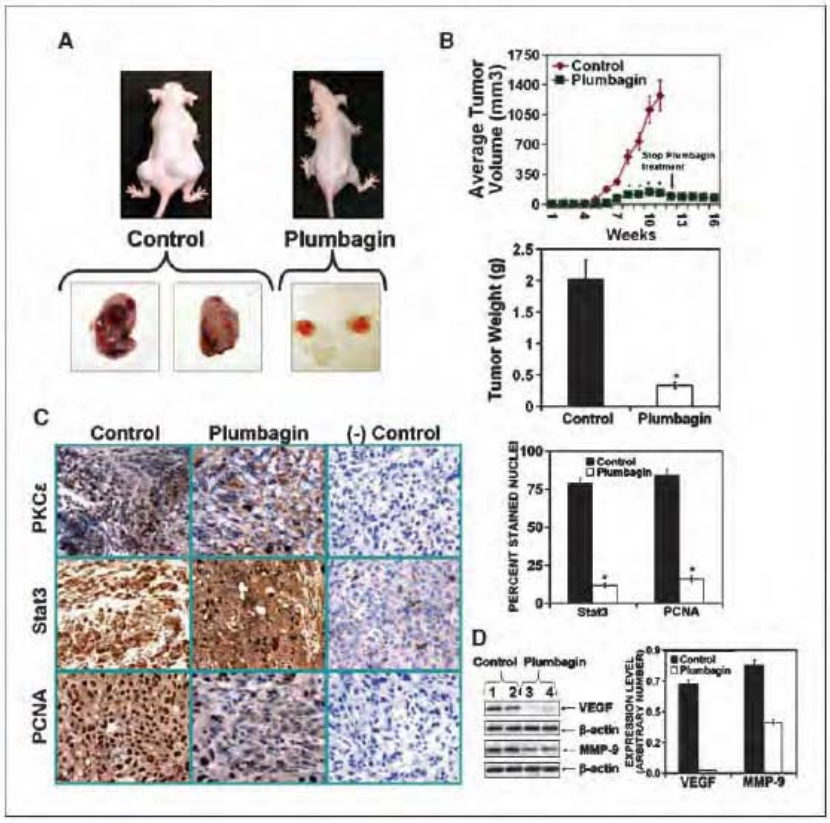

Figure 2. PL inhibits expression of PKCε, activated Stat3, PCNA, VEGF, and MMP-9 in ectopically xenografted DU145 cells. The DU145 cells (2.5×10^6 in 100 μL of a 1:1 mixture of medium/Matrigel) were implanted on both flanks of athymic nude mice ($n = 10$ mice per group). The animals were treated with PL (2 mg/kg body weight in PBS or PBS only, 5 d a week) by i.p. injection beginning at 3 d after cell implantation. At the end of the study, mice were sacrificed and digital photographs were taken. *A, top,* photographs of representative mice; *bottom,* photographs of excised tumors. *B, top,* tumor growth kinetics. Tumor growth was measured weekly using digital calipers and the average tumor volume was graphed as a function of time. After 11 wk, PL treatment was stopped and tumor growth was measured through 16 wk after cell implantation. *, $P < 0.05$, from the control group. *Bottom,* tumor weight at 11 wk after cell implantation. *C, left,* immunohistochemistry of tumor tissue for PKCε, Stat3, and PCNA with negative controls for specificity. Magnification, ×40 (*left*). *Right,* quantitation of Stat3 and PCNA-positive stained nuclei. *Columns,* mean of 10 different views; *bars,* SE. *, $P < 0.000$, from the control group. *D, left,* expression of VEGF and MMP-9 in tumors from PL-treated and control mice; *right,* quantitation of VEGF and MMP-9 expression.

Olympus BX51 microscope. Negative controls (without primary antibody) were included for each study. For the quantitation of Stat3 and PCNA-positive staining cells, 10 random areas were selected for each mouse at each time point. The number of cells showing positive labeling and the total number of cells counted were recorded. An average percentage was then calculated based on the total number of cells and the number of positive staining cells from each set of 10 fields counted. Results are expressed as mean of percentages ± SE.

Electrophoretic mobility shift assay. PCa cells (DU145, PC-3, CWR22rv1, and LNCaP) at 70% to 80% confluency were serum starved for 24 h. Cells were treated with 0, 5, 10, 15, or 20 μmol/L of PL for 3 h. Nuclear protein extracts were prepared by lysing cells in a hypotonic solution [10 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 0.1 mmol/L EDTA (pH 8.0), 0.1 mmol/L EGTA (pH 8.0), 1 mmol/L DTT, 0.5 mmol/L PMSF, 0.5 mg/mL benzamide, 2 μg/mL aprotinin, 2 μg/mL leupeptin], with detergent [NP40 at 6.25% (v/v)] followed by low speed ($1,500 \times g$ for 30 s) to collect nuclei. Nuclear proteins were extracted in a high-salt buffer [20 mmol/L HEPES (pH 7.5), 0.4 mol/L NaCl, 1 mmol/L EDTA (pH 8.0), 1 mmol/L EGTA (pH 8.0), 1 mmol/L DTT, 1 mmol/L PMSF, 0.5 mg/mL benzamide, 2 μg/mL aprotinin, 2 μg/mL leupeptin] and nuclear membranes and genomic DNA were removed by high-speed ($16,000 \times g$) centrifugation for 5 min. Nuclear protein extracts were stored at −70°C until used. The nuclear protein extract was incubated in a final volume of 20 μL of 10 mmol/L HEPES (pH 7.9), 80 mmol/L NaCl, 10% glycerol, 1 mmol/L DTT, 1 mmol/L EDTA, and 100 μg/mL poly(deoxyinosinic-deoxycytidylic acid) for 15 min. γ-^{32}P–radiolabeled double-stranded oligonucleotides of the consensus binding sequences of AP-1, NF-κB, or Stat3 were then added and the complexes were incubated for 20 min at room temperature. The protein-DNA complexes were resolved on a 4.5% acrylamide gel containing 2.5% glycerol and 0.5× Tris-borate EDTA at room temperature. Gels were dried and autoradiographed to determine binding activity (14).

Results

PL inhibits invasion and induces apoptosis in PCa cells. Cell invasion requires cells to migrate through an extracellular matrix or basement membrane barrier by first enzymatically degrading the barrier and then becoming established in a new location. Cell invasion is exhibited by tumor cells during metastasis. The effects of PL on the invasive ability of AI human PCa cell lines were determined. In this experiment (Fig. 1*C*), PCa cells (DU145, PC-3, and CWR22rv1) were treated with 5 or 20 μmol/L of PL for 48 h and cell invasion was assayed using a Collagen-Based Cell Invasion Assay kit (14). PL, at both 5 and 20 μmol/L concentration, significantly ($P < 0.001$) inhibited the invasion of DU145, PC-3, and CWR22rv1. The inhibitory effect of PL on cell invasion did not differ among these cell lines (DU145, PC-3, and CWR22rv1; $P > 0.1$; Fig. 1*C* and *D*). The effect of PL on the induction of apoptosis in human PCa has recently been reported (12). PL induced apoptosis in human PCa cells (PC-3, LNCaP, and C4-2) irrespective of androgen responsiveness and p53 status. PL-induced apoptosis in human PCa cells was associated with modulation of cellular redox status and generation of reactive oxygen species (ROS; ref. 12). We also determined the effects of PL on the induction of apoptosis in PCa cell lines (DU145, CWR22rv1, and LNCaP) and nontumorigenic

immortalized prostate epithelial RWPE-1 cells. PL at concentration as high as 20 μmol/L did not significantly ($P = 0.42$) induce apoptosis in RWPE-1 cells (Fig. 1*B*). PL at all concentrations significantly ($P < 0.009$) induced apoptosis in PCa cell lines (DU145, CWR22rv1, and LNCaP). AI PCa cells (DU145 and CWR22rv1) seem to be more sensitive than AD PCa cells (LNCaP) to the induction of apoptosis by PL (Fig. 1*B*).

PL inhibits growth of DU145 cells in athymic nude mice. In this experiment (Fig. 2*A* and *B*), PL (2 mg/kg body weight) was administered i.p. 3 days after ectopic implantation of hormone-refractory DU145 cells. PL treatment delayed tumor growth by 3 weeks and significantly ($P < 0.05$) reduced both the tumor weight and volume throughout the experimental period (Fig. 2*A* and *B*). Discontinuation of PL treatment in PL-treated mice, for as long as 4 weeks, did not result in an increase in tumor growth (Fig. 2*B*). PL treatment significantly ($P = 0.000$) inhibited PCNA expression and constitutive expression of Stat3 and PKCε (Fig. 2*C*). In addition, PL treatment inhibited the expression of VEGF and MMP-9 (Fig. 2*D*). The PL-treated mice gained weight and exhibited no obvious toxic effects.

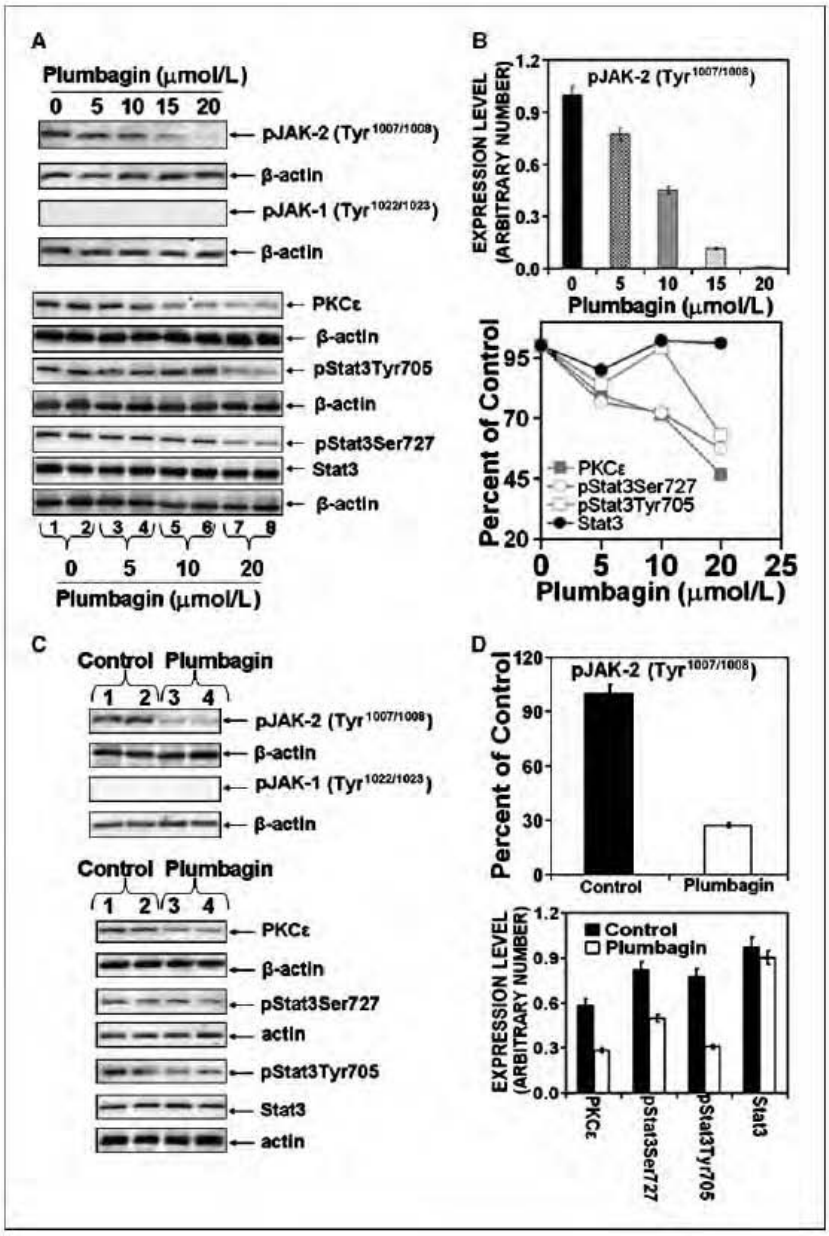

Figure 3. PL inhibits PKCε expression as well as JAK-2 and Stat3 phosphorylation in DU145 cells *in vitro* and *in vivo*. *A* and *B*, DU145 cells at 70% to 80% confluency were serum starved for 24 h. Cells were treated with 0, 5, 10, 15, or 20 μmol/L of PL in DMSO (final concentration, 0.1%) for 6 h. Whole-cell lysates were prepared and used for Western blot analysis of the indicated proteins. *C* and *D*, DU145 cells (2.5×10^6 in 100 μL in a 1:1 of medium/Matrigel) were implanted on both flanks of nude mice. Animals were treated with PL (2 mg/kg body weight in PBS or PBS only, 5 d a week) by i.p. injection beginning 3 d after implantation. At the end of the study, tumors from PL-treated or control mice were excised and whole-cell lysates were prepared. Protein extracts (25 μg protein) were immunoblotted and indicated proteins were detected with the appropriate antibodies. Protein levels were normalized to β-actin. Western blots (*A* and *C*) were quantitated (*B* and *D*) by densitometric analysis using TotalLab Nonlinear Dynamic Image analysis software.

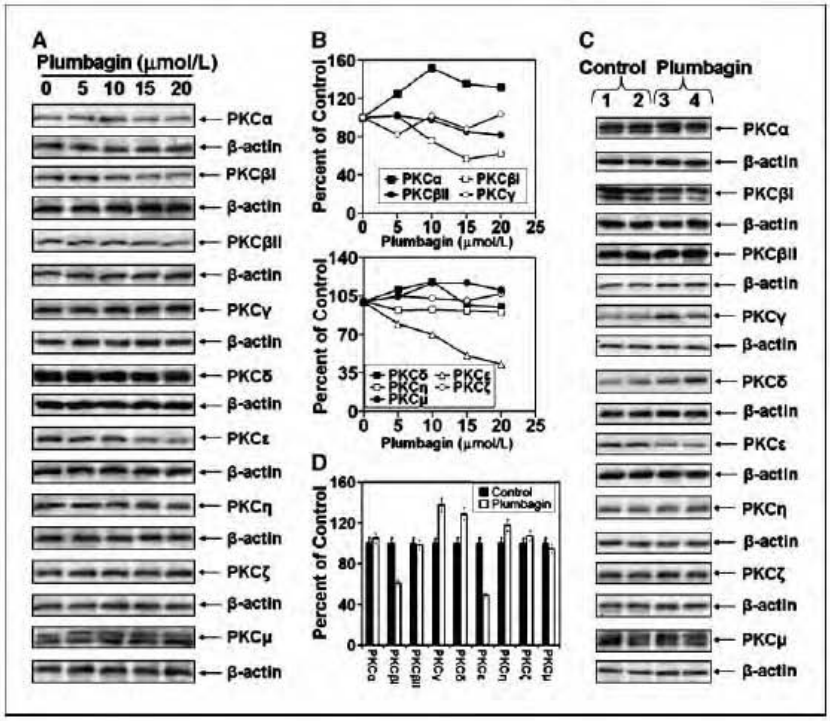

Figure 4. Effects of PL on the expression of PKC isoforms. DU145 cells at 70% to 80% confluency were serum starved for 24 h. Cells were treated with 0, 5, 10, 15, or 20 μmol/L of PL in DMSO (final concentration, 0.1%) for 6 h. *A*, whole-cell lysates were prepared and used for Western blot analysis of PKC isoforms. *C*, tumors from PL-treated or control mice were excised and whole-cell lysates were prepared to analyze the expression of PKC isoforms. *B* and *D*, quantitation of Western blots.

PL-induced inhibition of PCa cell growth accompanies inhibition of the expression of multiple molecular targets, including PKCε. To obtain clues about the mechanism by which PL may inhibit growth and invasion of PCa, we used both DU145 cells cultured *in vitro* and DU145 tumor xenografts from vehicle-treated and PL-treated mice. The results are illustrated in Fig. 3. PKCε expression and constitutive activation of Stat3 have been shown to play a role in the progression of human PCa (14). Stat3 activation, which involves dimerization, nuclear translocation, DNA binding, and transactivation of transcription, requires phosphorylation of both Tyr^{705} and Ser^{727} (14). Stat3Tyr705 phosphorylation is mediated by a wide variety of growth factors [e.g., interleukin-6 (IL-6)]. IL-6 signaling is mediated through JAK. JAK-Stat is the classic pathway that has been shown to mediate cellular responses to a variety of cytokines, including IL-6. In response to IL-6, Stat3 is transiently associated with gp130 and subsequently phosphorylated by JAKs on Tyr^{705} of Stat3. PKCε-mediated Stat3Ser727 phosphorylation is also essential for both optimal DNA-binding and transcriptional activities of Stat3 (14). A shown in Fig. 3*A* to *D*, PL treatment inhibited the expression of pJAK-2 and PKCε. PL-mediated inhibition of pJAK-2 and PKCε expression accompanied inhibition of both Stat3Ser727 and Stat3Tyr705 phosphorylation (Fig. 3*A*–*D*). The effects of PL on the expression of other PKC isoforms were also determined (Fig. 4). PL inhibited the expression of PKCε and PKCβ1. PKCα expression was slightly increased, whereas expression levels of other PKC isoforms (PKCβ, PKCγ, PKCδ, PKCη, PKCζ, and PKCμ) were unaffected (Fig. 4). Constitutively activated PKCε is linked to cell survival essential for maintenance of PCa. We observed in PCa from TRAMP mice that PKCε expression accompanied up-regulation of phosphorylated PI3K and AKT, major components of the cell survival pathway (14). These results prompted us to analyze the effects of PL on the expression of PI3K and AKT in DU145 cells and tumors. The results are shown in Fig. 5. PL treatment inhibited the expression of the PI3K (p85) and PI3K (p110) regulatory subunits and pAKT (Ser^{473} and Thr^{308}; Fig. 5*A* and *B*). We also observed that PL treatment induced the expression of p21 and p27 (Fig. 5*C* and *D*).

PL treatment indiscriminately inhibits the DNA-binding activity of transcriptional factors AP-1, NF-κB, and Stat3 in PCa cell lines. Activation of PKCε and PI3K/AKT pathways culminates in the activation of transcription factors (AP-1, NF-κB, and Stat3), which drive the expression of cell survival genes (14). Sandur and colleagues (1) have reported that PL-modulated cell proliferation, carcinogenesis, and radioresistance may be due to inhibition of NF-κB pathway. We found that PL inhibited the DNA-binding not only of NF-κB but also of AP-1 and Stat3 in PCa cell lines DU145, PC-3, and CWR22rv1 (Fig. 6*A*). Inhibition of the DNA-binding activity was observed at PL concentrations as low as 5 μmol/L (Fig. 6*A*). Figure 6 also shows that PL inhibited the expression of several cell survival genes (*COX-2*, *cdc25A*, and *Bcl-xL*; Fig. 6*C* and *D*).

Discussion

PCa is the most common type of cancer in American men and ranks second to lung cancer in cancer-related deaths (1). Hormone-refractory invasive PCa is the end stage and accounts for the majority of PCa patient deaths (2–7). Men with hormone-refractory cancer are at high risk for developing bone metastasis, which results in clinically significant skeletal morbidity (15–18). The

management of locally advanced PCa is difficult and complex because the cancer often becomes unresponsive to current chemotherapeutic agents. Several agents, such as selenium, lycopene, soy products, green tea, pomegranate phenolics, apigenin, and vitamins D and E, are effective in the prevention of the induction of PCa (19–22). However, there is no agent that is in fact effective and selective in the prevention and/or treatment of late-stage hormone-refractory PCa. We present here that PL, a quinoid constituent isolated from the roots of medicinal plant *Plumbago zeylanica L.* (also known as Chitrak; ref. 7), induces apoptosis and inhibits invasion of AI PCa cells (Fig. 1). Administration of PL (2 mg/kg body weight), beginning 3 days after ectopic implantation of hormone-refractory DU145 PCa cells, delays tumor growth by 3 weeks and reduces both tumor weight and volume by 90% (Fig. 2). In addition, PL abrogates the expression of PKCε (Fig. 3), which plays a role in the development and maintenance of AI PCa (14).

The results (Fig. 1) involving the induction of apoptosis in PCa cells by PL are consistent with findings using other cancer cell lines, such as ovarian cancer BG1 cells (23), cervical cancer cells (24), and breast cancer cells (9). PL-induced apoptosis involves G_2-M arrest and generation of ROS (10). ROS-mediated inhibition of topoisomerase II has been suggested to be a mechanism contributing to the apoptosis-inducing properties of PL (25). It is also noteworthy that PL in breast cancer cell lines has been reported to trigger autophagic cell death but not predominantly apoptosis (9).

We provide direct experimental evidence that PL has efficacy in preclinical model of ectopic growth of PCa cells in nude mice (Fig. 2). Inhibition of tumor growth may be the result of inhibition of the expression of cell proliferative marker PCNA as well as inhibition of the constitutive activation of cell survival markers PKCε and Stat3 (Fig. 3).

Metastasis is the primary cause of mortality from cancer (15–18). Cell migration and invasion play critical roles in cancer metastasis (15–18). PL was observed to be a potent inhibitor of PCa cell invasion (Fig. 1). The molecular mechanism linked to PL-induced inhibition of PCa cell invasion may involve inhibition of the expression of MMP-9 and VEGF (Fig. 1), the components in cell invasion and metastasis (26–28).

PL inhibits PKCε expression and Stat3 activation (Figs. 3 and 4). PKCε is a member of the novel PKC subfamily (29–33). PKCε is an important component of the mechanism of induction and progression of PCa (14). PKCε is overexpressed in human PCa and PCa developed either in C57BL/6 or [C57BL/6 × FVB] F1 TRAMP mice (14). The fact that PKCε expression is significantly elevated in PCa and correlates with PCa aggressiveness (14, 34) implies that PKCε is probably linked to the maintenance of AI PCa. In this context, the pioneering work of Terrian and his associates on the role of PKCε in prostate carcinogenesis, using PCa-derived cell lines, is noteworthy (34–37). In their reports, PKCε overexpression transformed AD LNCaP tumor cells to AI cells (35). The transformation of AD LNCaP cells to an AI variant was associated with increased cell proliferation and resistance to apoptosis. Antisense experiments established that endogenous PKCε plays an important role in regulating the growth and survival of AI PCa cells, suggesting that PKCε expression may be sufficient to

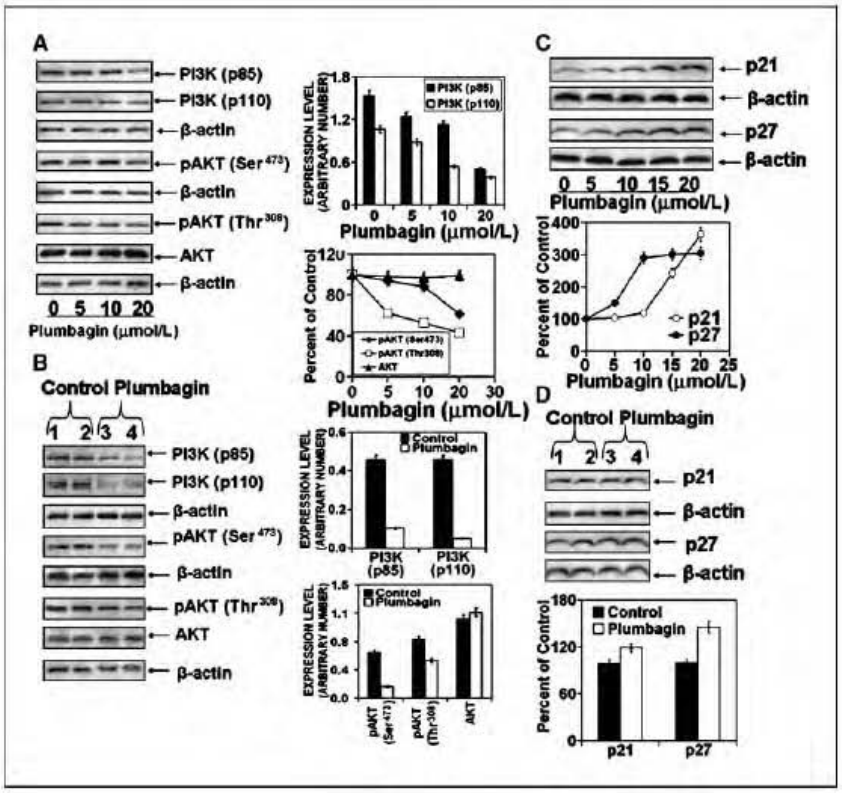

Figure 5. Effects of PL on the expression PI3K, AKT, p21, and p27. DU145 cells at 70% to 80% confluency were serum starved for 24 h. Cells were treated with 0, 5, 10, 15, or 20 μmol/L of PL in DMSO (final concentration, 0.1%) for 6 h. *A* and *C*, whole-cell lysates were prepared and used for Western blot analysis of PI3K, AKT, p21, and p27. *B* and *D*, tumors from PL-treated or control mice were excised and whole-cell lysates were prepared to analyze the expression PI3K, AKT, p21, and p27.

maintain PCa growth and survival after androgen ablation (35). PKCε is a transforming oncogene and a predictive biomarker of breast cancer and PCa (14).

PKCε associates with Stat3 and regulates Stat3 activation. Stat3 is activated by phosphorylation at both Tyr^{705} and Ser^{727} residues. Constitutively activated Stats, particularly Stat3, have been found in several human cancers (e.g., squamous cell carcinomas, head and neck, breast, ovary, prostate, and lung; refs. 38–45). PKCε activation transduces multiple signals involving inhibition of apoptotic pathways and promotion of cell survival pathways. PKCε-mediated cell survival pathway involves constitutive activation of Stat3. PKCε is an initial signal that regulates activation of Stat3. PKCε may be a primary target of PL for prevention of AI PCa progression.

PL inhibits the activation of PI3K/AKT (Fig. 5*A* and *B*). As observed in PCa from TRAMP mice, PKCε expression accompanied up-regulation of phosphorylated PI3K and AKT, major components of the cell survival pathway (14). Consistent with these findings, using CWR22 xenografts, it was shown by proteomic analysis that the association of PKCε with Bax may neutralize apoptotic signals propagated through the mitochondrial death signaling pathway (46). In addition, integrin signaling links PKCε to the PKB/AKT survival pathway in recurrent PCa cells (34). PL inhibits PKCε overexpression, which correlates with PCa aggressiveness and accompanies an increase in proteins that modulate apoptosis (survivin, Bcl-2, and Bcl-xL), and cell cycle progression (p21 and p27; Fig. 5*C* and *D*).

It is notable that Sandur and colleagues (7) reported that PL is a specific inhibitor of NF-κB and does not suppress activation of other transcription factors AP-1 and Stat3 in KBM-5 (human chronic myeloid leukemia) and U266 (human multiple myeloma) cells. The discrepancy between our results with the PCa cell lines (PC-3, DU145, and CWR22rv1) and their results with KBM-5 and U266 cells may be due to cellular context.

In several repeat experiments, PL inhibited the constitutive activation of AP-1, NF-κB, and Stat3 in AI PCa cell lines PC-3, DU145, and CWR22rv1 but not in AD PCa cell line LNCaP. These results indicate that androgen receptor (AR) status may determine

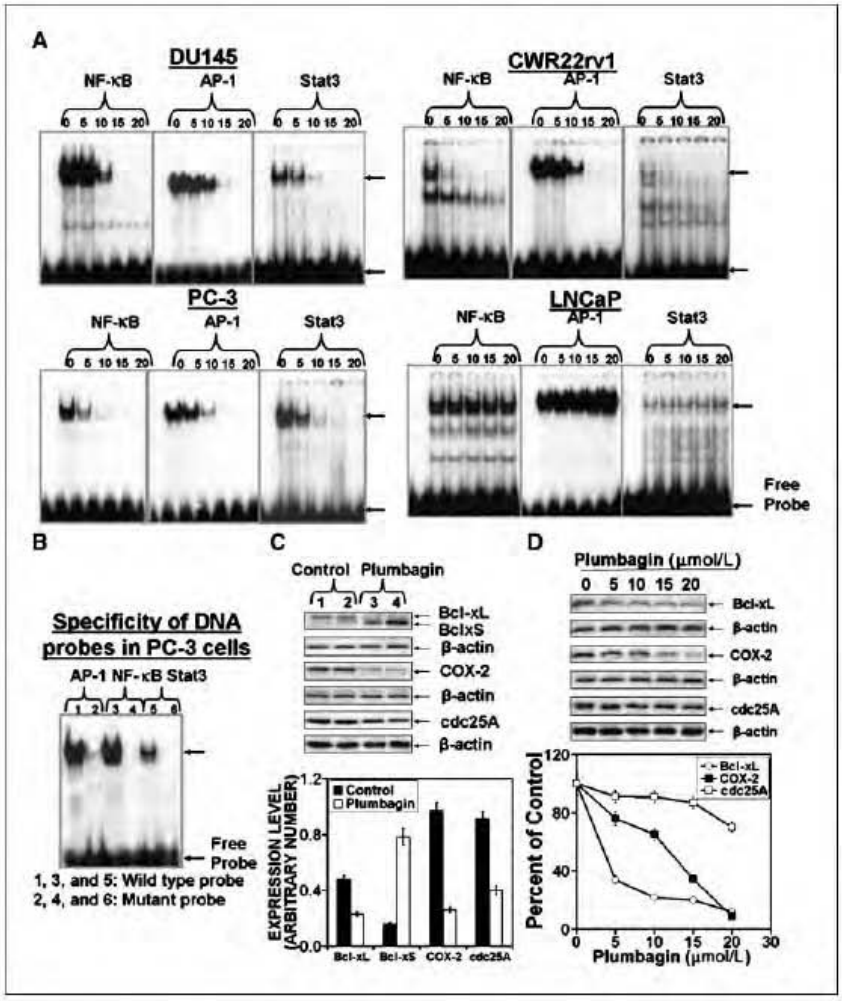

Figure 6. PL inhibits DNA binding of transcription factors Stat3, NF-κB, and AP-1 and transcription factor–regulated gene expression. PCa cells (DU145, PC-3, CWR22rv1, and LNCaP) at 70% to 80% confluency were serum starved for 24 h. Cells were treated with 0, 5, 10, 15, or 20 μmol/L of PL in DMSO (final concentration, 0.1%) for 3 h. Nuclear protein extracts were prepared by lysing cells in a hypotonic solution [10 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 0.1 mmol/L EDTA (pH 8.0), 0.1 mmol/L EGTA (pH 8.0), 1 mmol/L DTT, 0.5 mmol/L PMSF, 0.5 mg/mL benzamide, 2 μg/mL aprotinin, 2 μg/mL leupeptin], with detergent [NP40 at 6.25% (v/v)] followed by low speed centrifugation (1,500 × *g* for 30 s) to collect nuclei. Nuclear proteins were extracted in a high-salt buffer [20 mmol/L HEPES (pH 7.5), 0.4 mol/L NaCl, 1 mmol/L EDTA (pH 8.0), 1 mmol/L EGTA (pH 8.0), 1 mmol/L DTT, 1 mmol/L PMSF, 0.5 mg/mL benzamide, 2 μg/mL aprotinin, 2 μg/mL leupeptin] and nuclear membranes and genomic DNA were removed by high-speed centrifugation. Nuclear protein extracts were stored at −70°C until used. *A*, electrophoretic mobility shift assay of NF-κB, AP-1, and Stat3 DNA binding. *B*, specificity of AP-1, NF-κB, and Stat3 DNA binding. *C* and *D*, transcription factor–regulated gene expression. *C*, tumors from PL-treated or control mice were excised and whole-cell lysates were prepared to analyze the expression of indicated proteins. *D*, DU145 cells at 70% to 80% confluency were serum starved for 24 h. Cells were treated with 0, 5, 10, 15, or 20 μmol/L of PL for 6 h. Whole-cell lysates were prepared and used for Western blot analysis of indicated proteins. *Bottom*, quantitation of Western blots *C* and *D*.

PL-induced suppression of transcription factors AP-1, NF-κB, and Stat3. The mechanism by which PL suppresses the constitutive activation of AP-1, NF-κB, and Stat3 in AI PCa cells is unclear. However, PL inhibits constitutive expression of PKCε, which may play a role in the activation of AP-1, NF-κB, and Stat3.

The role of PKCε in PL-induced inhibition of growth and invasion of AI PCa is speculative. Most AI PCa continue to express AR as well as the AD gene *PSA*, which indicates that these cells maintain a functional AR signaling pathway despite castrate levels of testosterone. Gene amplification and mutations in AR are frequently observed in recurrent PCa, which may account for hypersensitivity of the AR to low castrate level of androgens, and altered ligand specificity (47). Increased AR activity in AI PCa is perhaps caused by cross-talk of AR with multiple intracellular signaling cascades, including peptide growth factors [epidermal growth factor (EGF), transforming growth factor-β, and insulin-like growth factor-I; ref. 48]. In this context, it is noteworthy that HER-2/neu, a member of the EGF family of receptor tyrosine kinases, activates the AR pathway in the absence of ligand (49). It remains to be determined whether there is cross-talk between AR and PKCε signal transduction pathway in the progression of AI PCa.

PL has also been extensively evaluated for toxic side effects in rodents. Toxic side effects included diarrhea, skin rashes, and hepatic and reproductive toxicity. These toxic side effects were dose related. The LD_{50} for these side effects in mice was 8 to 65 mg/kg body weight for p.o. administration and 16 mg/kg body weight for i.p. (7). PL has been reported to be nontoxic at doses (2 mg/kg body weight i.p. or 200 ppm in diet) shown to elicit chemopreventive and therapeutic effects (7). In addition, the mutagenic activity of PL in *Escherichia coli* has been examined and was negative in the Ames test (7).

In summary, PL, a plant-derived naphthoquinone, inhibits the growth and invasion of AI PCa cells (Figs. 1 and 2). PL-induced inhibition of PCa cell growth and invasion accompanies inhibition of multiple targets, including PKCε and transcription factors AP-1, NF-κB, and Stat3 (Figs. 3–6). The results (Figs. 1–6) presented have led us to propose that PKCε is a master switch in the progression and invasion of hormone-refractory PCa. PKCε directly or indirectly via association with other protein kinases [e.g., Raf-1, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1/2, ERK1/2, and p38MAPK] phosphorylates Stat3Ser727. Constitutive activation of PKCε and Stat3 is correlated with the aggressiveness of PCa (14). PI3K/PKD3/AKT may phosphorylate AR, enabling to form dimers, thus enhancing AR-DNA binding and gene expression (50). We hypothesize that PL inhibits the expression of PKCε, an initial signal in the development of AI PCa.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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